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<p>(54) Title: PURIFICATION OF OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES USING DEAE SPW ANION ION-EXCHANGE CHROMATOGRAPHY AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY</p>		
<p>(57) Abstract</p> <p>The present invention provides improved methods for separating and purifying oligonucleotide phosphorothioates. In certain aspects of the invention, oligonucleotide phosphorothioates are purified by a method comprising applying a mixture of the oligonucleotide to a column containing a DEAE-SPW anion ion-exchange chromatography resin and eluting with an elution buffer having a concentration of sodium chloride of from about 0-2M. In other aspects of the invention, oligonucleotide phosphorothioates are purified by a method comprising applying a mixture of the oligonucleotide to a column containing a phenyl-sepharose fast flow chromatography resin or a phenyl-5PW chromatography resin and eluting with an elution buffer substantially free of salts. The methods of the present invention provide the ability to purify ammoniacal solutions of oligonucleotides cheaply, quickly, and on a large, process scale.</p>		

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**PURIFICATION OF OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES  
USING DEAE 5PW ANION ION-EXCHANGE CHROMATOGRAPHY AND  
HYDROPHOBIC INTERACTION CHROMATOGRAPHY**

**BACKGROUND OF THE INVENTION**

**5    Field of the Invention**

This invention relates to the field of purification of oligodeoxynucleotides, and in particular, the purification of oligodeoxynucleotide phosphorothioates.

**Description of the Prior Art**

The use of modified phosphate backbone oligodeoxynucleotides as antisense  
10    oligonucleotides in the field of selective gene regulation for therapeutic purposes has  
received increasing attention over the last several years. There are numerous types of  
modified phosphate linkages, e.g., methylphosphonate, phosphorothioate,  
phosphoramidate, that have been incorporated into antisense oligonucleotides and studied.  
E.g., Erickson and Izant (Eds.), *Gene Regulation: Biology of Antisense RNA and DNA*  
15    (Raven Press, New York, 1992). Oligodeoxyribonucleotide phosphorothioates, for  
example, have been found to inhibit immunodeficiency virus (Agrawal et al., *Proc. Natl.*  
*Acad. Sci. USA* 85, 7079 (1988); Agrawal et al., *Proc. Natl. Acad. Sci. USA* 86, 7790  
(1989); Agrawal et al., in *Advanced Drug Delivery Reviews* 6, 251 (R. Juliano, Ed.,  
Elsevier, Amsterdam, 1991); Agrawal et al. in *Prospects for Antisense Nucleic Acid*  
20    *Therapy of Cancer and AIDS*, 143 (E. Wickstrom, Ed., Wiley/Liss, New York, 1991); and  
Zamecnik and Agrawal in *Annual Review of AIDS Research*, 301 (Koff et al., Eds.,  
Dekker, New York, 1991)), and influenza virus (Leiter et al., *Proc. Natl. Acad. Sci. USA*  
87, 3420-3434 (1990)) in tissue culture. In addition, oligodeoxyribonucleotide  
phosphorothioates have been the focus of a wide variety of basic research (e.g., Agrawal  
25    et al., *Proc. Natl. Acad. Sci. USA* 87, 1401 (1990) and Eckstein and Gish, *Trends*  
*Biochem. Sci.* 14, 97 (1989)), enzyme inhibition studies (Mujumdar et al., *Biochemistry*  
28, 1340 (1989)), regulation of oncogene expression (Reed et al., *Cancer Res.* 50, 6565  
(1990)) and IL-1 expression (Manson et al., *Lymphokine Res.* 9, 35 (1990)) in tissue  
culture.

30    Automated synthesizers have proven an invaluable tool for obtaining  
oligonucleotides. Oligonucleotides are produced stepwise, with the addition of one  
monomer at a time to the nascent oligonucleotide chain. 2-3% of the reactions fail during  
each cycle in which a nucleotide monomer is to be added however. Consequently, the

resulting products are generally a heterogenous mixture of oligonucleotides of varying length. For example, in a typical 20mer synthesis, the 20mer product represents only 50-60% of the recovered oligonucleotide product.

Furthermore, preparation of oligodeoxynucleotides on a solid phase support  
5 requires that the oligodeoxynucleotide be cleaved from the support. Cleavage of the oligo from the support is typically accomplished by treating the solid phase with concentrated ammonium hydroxide. The ammonium hydroxide is conventionally removed under reduced pressure using, for example, a rotary evaporator. This method for removing the ammonium hydroxide, however, is not ideal for use in large scale isolation of  
10 oligodeoxynucleotides.

For most purposes (e.g., therapeutic or diagnostic) the purity of the compounds is extremely important. Consequently, there has been an interest in developing chromatographic techniques for purifying oligonucleotides. Because of their therapeutic potential, much of the focus has been on purifying oligonucleotide phosphorothioates.

15 Conventional methods for purifying oligodeoxynucleotides employ reverse-phase liquid chromatography. Such methods require explosion-proof equipment because acetonitrile is typically used in the elution buffer.

Methods of oligodeoxynucleotide phosphorothioate purification have been published. Agrawal et al., *J. Chromatography* 509, 396 (1990), reported the analysis of  
20 oligonucleotide phosphorothioates using high-performance liquid chromatography with a reverse-phase column. In that study, Agrawal et al. converted the oligonucleotide phosphorothioate to its phosphodiester counterpart and then carried out HPLC analysis. Using this method they were able to analyze oligonucleotide phosphorothioates containing 10 or fewer nucleotides on a strong anion-exchange column (Partisphere SAX column).  
25 Oligonucleotide phosphorothioates having more than 10 nucleotides could not be analyzed, however, because of the strong interaction with the SAX medium.

Metlev and Agrawal, *Anal. Biochem.* 200, 342 (1992), reported the ion-exchange HPLC analysis of oligodeoxy-ribonucleotide phosphorothioates on a weak anion-exchange column (Partisphere WAX) in which the weak anion exchanger utilizes a  
30 dimethylaminopropyl functional group bonded to Partisphere silica. This medium, with an ion-exchange capacity of 0.18 meq/g, exhibits an interaction with anions weaker than those observed with strong anion-exchange media. The authors of this study found that

separation was length dependent for oligonucleotide phosphorothioates up to 25 nucleotides in length. Furthermore, n-1 peaks were well separated from the parent peak. They also found that 30-mer and 35-mer oligonucleotide phosphorothioates were separable with the same gradient, although better separation could be obtained with a shallower  
5 gradient.

Metel'ev et al., *Ann. N.Y. Acad. Sci.* **660**, 321-323 (1992), reported the analysis of oligoribonucleotides and chimeric oligoribo-oligodeoxyribonucleotides using ion-exchange HPLC. They found that the retention time of the oligonucleotides studied depended on the number of ribonucleotide moieties in the oligonucleotide. In addition, the retention time  
10 of oligoribonucleotides was found to be length dependent. The authors noted that oligoribonucleotides of length up to 25 nucleotides could be purified and analyzed.

Bigelow et al., *J. Chromatography* **533**, 131 (1990), reported the use of ion-pair HPLC to analyze oligonucleotide phosphorothioates. Stec. et al., *J. Chromatography* **326**, 263 (1985), and Agrawal and Zamecnik, *Nucleic Acids Res.* **19**, 5419 (1990), reported  
15 HPLC analysis of oligodeoxyribonucleotides containing one or two phosphorothioate internucleotide linkages using a reversed-phase column.

These methods of oligonucleotide phosphorothioate purification use HPLC. While this technique is useful for small scale operations, it is unsuitable for large, commercial scale use. Consequently, improved methods of oligonucleotide purification suitable for  
20 use in large scale oligonucleotide preparations is desirable.

### **SUMMARY OF THE INVENTION**

The present invention provides improved methods for purifying oligodeoxynucleotide phosphorothioates. In particular, the invention provides purification techniques suitable for large scale separation of oligonucleotide phosphorothioates. The  
25 purification methods of the invention do not require the use of reduced pressure to remove ammonium hydroxide or the use of conventional C-18 silica gel reverse-phase liquid chromatography. The inventive methods replace these procedures with hydrophobic interaction chromatography or DEAE-5PW anion ion-exchange chromatography.

In one aspect of the present invention, the oligodeoxynucleotides are purified using  
30 hydrophobic interaction chromatography. Ammonium hydroxide is used to cleave oligonucleotides from the solid support on which they were synthesized. Typically, roto-

evaporation under reduced pressure has been used to remove most of the ammonium hydroxide. This is then typically followed with reversed phase chromatography to separate the DMT-on oligonucleotides from the DMT-off oligonucleotides. These techniques, however, are unsuitable for large scale use. In this aspect of the invention, 5 hydrophobic interaction chromatography is used in place of roto-evaporation alone or both roto-evaporation *and* reversed phase chromatography. HIC is preferable to roto-evaporation because it simplifies and accelerates ammonium hydroxide removal and can be used for large scale purification. If it is to be followed by RPLC, HIC increases the purity of the oligonucleotide relative to roto-evaporation, resulting in less potential for 10 fouling the RPLC column and reducing the purification challenge presented to the RPLC column.

When used in place of roto-evaporation and RPLC, HIC provides the benefit of accomplishing two tasks (removal of ammonium hydroxide and separation of DMT-on oligos from DMT-off oligos) at once. Substitution of RPLC with HIC also reduces the 15 resin cost, eliminates the need for organic solvents (which require more stringent handling, including special disposal, explosion-proof environment, and evaporative equipment), provides for more rapid elimination of contaminants (e.g., unreacted monomers and failure sequences), and increases throughput. This increase in throughput is made possible by use of short columns and high linear velocities.

20 HIC also reduces both the expense (in terms of column packing and equipment) and potential problems that can arise with HPLC, e.g., difficulties in packing and maintaining HPLC columns. Suitable HIC columns that can be used in the present invention include, but are not limited to, phenyl-sepharose fast flow (high substitution) and TSK-gel phenyl-5PW.

25 Interestingly, although HIC and DEAE-5PW chromatography are mechanistically quite different, they can be used interchangeably to serve the same purpose. They both can be used to purify DMT-off oligonucleotides, although, as demonstrated below, DEAE-5PW results in better yields when purifying 25mers. Oligonucleotide mixtures having purities of about 98% can regularly be obtained using these techniques.

30 The use of DEAE-5PW column to purify DMT-off oligonucleotides, like HIC columns, does not require HPLC and, therefore, offers the same advantages as described

accomplished on a relatively short column, which increases throughput and eases packing, and requires simple step gradients for elution, which simplifies equipment requirements and the chance for error.

In yet another aspect of the present invention, ion-exchange is accomplished with a DEAE-5PW column. When oligonucleotides intended for therapeutic use, it is essential that all ammonium cations be replaced with, for example sodium cations. This can be accomplished with a Dowex cation ion exchange column followed by desalting with sephadex gel filtration. In this aspect of the invention, standard ion-exchange methods are replaced by the DEAE-5PW column. The resin is relatively cheap compared to the more recently introduced anion ion-exchange styrene divinylbenzene polymer supports (e.g., PerSeptive Biosystems, Polymer Labs), yet is sturdy enough (in terms of particle size and resistance to currently used cleaning procedures) for production use.

When the oligodeoxynucleotides are purified using a DEAE-5PW resin, the eluate generally has a very high salt concentration, rendering typical sephadex gel filtration desalting impractical or impossible. In place of sephadex gel filtration, other salt removal techniques, e.g., RPLC and tangential flow filtration (TFF), should be used. In the preferred embodiment, the DEAE-5PW oligonucleotide mixture is desalted using tangential flow filtration.

The foregoing merely summarizes certain aspects of the present invention and is not intended, nor should it be construed to limit the invention in any way.

All patents and publication cited in this specification are hereby incorporated by reference in their entirety.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides improved methods for purifying oligodeoxynucleotide phosphorothioates. In particular, the invention provides purification techniques suitable for large scale separation of oligonucleotide phosphorothioates. The purification methods of the invention do not require the use of reduced pressure to remove ammonium hydroxide or the use of conventional C-18 silica gel reverse-phase liquid chromatography. The inventive methods replace these procedures with hydrophobic interaction chromatography or DEAE-5PW anion ion-exchange chromatography.



Following solid phase synthesis, oligonucleotides are cleaved from the solid support by incubating the support in ammonium hydroxide. Not only are the desired oligonucleotides cleaved from the support, but so too are failure sequences, i.e., oligonucleotide sequences being fewer than the desired number of nucleotides in length. Such failure sequences arise from less than complete coupling of mononucleosides to the growing oligonucleotide chain and less than complete capping of unreacted functional sites. The desired oligonucleotide must be separated from failure sequences if it is to be used effectively for therapeutic or other purposes.

Conventionally, the bulk of the ammonium hydroxide is driven off by roto-evaporation. This is then followed by separating the desired DMT-on oligos (i.e., oligonucleotides having a 5' dimethoxytrityl protecting group) from undesired DMT-off oligos (i.e., oligonucleotides not having a 5' protecting group) by reversed phase liquid chromatography. The DMT-on oligos, being more hydrophobic, bind to the reverse phase column more tightly than DMT-off oligos.

If the desired oligonucleotide is to be used for therapeutic purposes, any ammonium cations complexed with the oligonucleotide must be exchanged with, for example, sodium cations. This is generally accomplished by ion exchange chromatography followed by desalting using sephadex gel.

The present invention comprises improved methods for separating or purifying oligonucleotide phosphorothioates. As used herein, the terms "separating" and "purifying" are intended to be used interchangeably and mean a process by which oligonucleotides having a particular molecular structure are physically segregated from oligonucleotides having a different molecular structure and by which ammonium hydroxide and/or other salts are removed from the oligonucleotide mixture. In particular, the present invention comprises the use of hydrophobic interaction chromatography (HIC) and DEAE-5PW ion exchange chromatography to separate and purify oligonucleotide phosphorothioates.

In a first embodiment of the present invention, the desired oligonucleotide is separated from excess ammonium hydroxide and prepared for treatment by reverse phase liquid chromatography by subjecting the oligonucleotide-containing ammonium hydroxide solution to hydrophobic interaction chromatography. Preferably, the HIC comprises

passing the oligonucleotide-containing ammonium hydroxide through a phenyl-sepharose fast flow chromatography resin or a phenyl-5PW chromatography resin.

In a second embodiment of the present invention, the desired oligonucleotide is separated from excess ammonium hydroxide and DMT-off oligos by subjecting the  
5 oligonucleotide-containing ammonium hydroxide solution to hydrophobic interaction chromatography. Preferably the HIC comprises passing the oligonucleotide-containing ammonium hydroxide through a phenyl-sepharose fast flow chromatography resin or a phenyl-5PW chromatography resin.

In a third embodiment of the present invention, ion exchange (wherein ammonium  
10 ions are exchanged for sodium ions) and purification are accomplished with a DEAE-5PW column. In this embodiment it is found that salt concentrations of the eluant are generally too high for desalting with sephadex-gel, as is traditionally done after standard oligonucleotide ion-exchange is conducted. In this embodiment, desalting is accomplished by any suitable technique capable of effectively handling high salt concentrations.  
15 Desalting is preferably conducted by tangential flow filtration (TFF). TFF provides the advantage of being able to accommodate and desalt large volumes of solutions having very high salt concentrations (e.g., 2 M NaCl).

The invention also encompasses a process for preparing purified mixtures of oligonucleotides. This aspect of the invention comprises:

- 20 (a) HIC to remove ammonium hydroxide and to remove oligos not having DMT protecting groups;  
(b) detritylation to remove the DMT protecting groups;  
(c) anion exchange on DEAE-5PW;  
(d) tangential flow filtration; and  
25 (e) lyophilization.

The methods of the invention may be used to purify crude mixtures containing oligonucleotide phosphorothioates that have just been cleaved from a solid phase synthesis support using ammonium hydroxide or mixtures that have been previously subjected to a purification procedure but that are not of acceptable purity.

30 We have found that oligonucleotide phosphorothioates can be purified on DEAE-5PW ion-exchange columns, phenyl-5PW and phenyl-sepharose columns using aqueous

5PW results in effective separation of oligonucleotide phosphorothioates from phosphodiester in high yields. The protocols presented herein can be employed in large scale purification of oligonucleotide phosphorothioates to replace the conventional rotoevaporation and C<sub>18</sub> silica gel protocols required during the purification process. This results in fewer steps in the manufacturing process and allows for better purity and recovery of product.

According to the methods of the present invention, oligonucleotide phosphorothioates from about 10 to about 35 nucleotides in length can be separated on a DEAE-5PW ion exchange column, a phenyl sepharose column or phenyl-5PW column. In a preferred embodiment, oligonucleotide phosphorothioates having a length of from about 20 to about 35 and more preferably 25-30, can be separated using the present methods.

According to the invention, the oligonucleotides separable by the present method may have as few as one and as many as all phosphorothioate internucleotide linkages. As used herein, the term "oligonucleotide phosphorothioate" is used to describe such an oligonucleotide. Oligonucleotide phosphorodithioates of the same size as the oligonucleotide phosphorothioates described can also be separated by the inventive methods.

The oligonucleotides are placed on the column and eluted at or near ambient (room) temperature with either a gradient or a non-gradient buffer. For purification/preparation of ammoniacal solutions by HIC, ammonium acetate is used in equilibration buffers at concentrations ranging from about 0.5 to about 2.0 M, preferably 0.75 M. Although addition of ammonium acetate may reduce the pH somewhat, the pH should typically be high to minimize the loss of the trityl group. pH values of 7.5 - 11.0 have been used successfully. A typical value of 10.0 is preferred since this more alkaline pH minimizes loss of the trityl group from the oligonucleotide. Elution is most effectively accomplished with water, although other schemes employing buffers may prove useful in particular applications. Organic solvents are not required for separation, although their inclusion may be desirable when preparing oligonucleotides by HIC for subsequent RPLC separation. While taller columns can be used (*see* the height:diameter ratios in the Examples, *infra*), shorter columns work very well and are preferred for large-scale work. Column loads can go as high as 650 OD units/ml packing, depending on elution conditions

and column geometry. Preferably the column load is about 200 OD units/ml packing. Linear velocities can range as high as 300 cm/hr, but are preferably about 150 cm/hr.

When HIC is used to purify DMT-off oligonucleotides, a variety of buffers can be used at a pH ranging from about 7.2 to about 8.5. In the preferred embodiment Tris-HCl is used at a pH of about 7.5. Salts such as sodium chloride must be added to the load and to the equilibration and wash buffers in concentrations ranging from about 1 M to about 3 M, preferably about 3 M. Elution is accomplished by either linear or step gradients via salt reduction. Phenyl sepharose columns are preferably loaded at high flow rates. Rates exceeding about 250 cm/hr are found to work exceedingly well. Elution at a rate of about 150-200 cm/hr is desirable.

When DEAE-5PW is used to purify DMT-off oligonucleotides, buffers such as Tris-HCl (preferably in a concentration of about 10 to about 50 mM) are to be used. Salts such as sodium chloride are used for equilibration and elution. Preferably, sodium chloride gradients of 0 to 2 M are used. For shorter columns a range of sodium chloride concentration between 0.85 and 2 M are preferred. The pH of the oligonucleotide solution that is loaded may be between about 7.0 and about 10.5, preferably about 7.2. The pH of equilibration, wash and elution buffers can range from about 7.2 to about 8.5, and is preferably about 7.2. Chelating agents such as 1 mM EDTA and organic solvents such as acetonitrile or ethanol can be added to the buffers in some applications. While linear gradients can be run, excellent recoveries and purities are obtained using simple step gradients. In many cases, better recoveries can be obtained using step gradients -- linear gradients appear to cause a slow "bleed-off" of bound product from the column that often works against effective separation and high recovery. While taller columns can be used, relatively short columns provide excellent results and are preferable for large-scale work. Column capacity for oligonucleotides is somewhat lower than that seen for the HIC resins, with optimum column loads ranging up to about 200 OD units/ml resin, preferably about 150 OD units/ml packing. Optimum linear velocities range between about 50 and about 150 cm/hr and are preferably about 70 cm/hr.

Column geometry has a significant effect on the requirements for equilibration buffer salt conditions -- taller columns (larger height:diameter ratio) require higher salt as compared to shorter columns (lower height:diameter ratio). When a mixture of

the relationship between the column height:diameter ratio and sodium chloride concentration is relevant to product yield. As the height:diameter ratio of the DEAE-5PW column is increased, the sodium chloride concentration of the equilibration buffer should preferably be increased to obtain a greater recovery of purified product.

5       The separation method of the present invention is essentially independent of column particle size. Sizes ranging from 25 to 90  $\mu\text{m}$  can be used successfully. In preferred embodiments, the particle size is 25  $\mu\text{m}$  - 40  $\mu\text{m}$  or 45  $\mu\text{m}$  - 165  $\mu\text{m}$ . A particularly preferred hydrophobic interaction chromatography resin has a particle size of about 90  $\mu\text{m}$ .

10       One skilled in the art will recognize that modifications may be made in the present invention without deviating from the spirit or scope of the invention. The invention is illustrated further by the following examples which are not to be construed as limiting the invention or scope of the specific procedures described herein.

### EXAMPLES

15       In each of the following Examples, equipment and materials were obtained as indicated from TosoHaas (Montgomeryville, PA), Amicon (Beverly, MA) Waters (Milford, MA), Hewlett-Packard (HP) (Palo Alto, CA), Perkin-Elmer (Norwalk, CT), ISCO (Lincoln NB), Rainin (Woburn, MA), Filtron (Northborough, MA), Pharmacia (Piscataway, NJ), and Biorad (Hercules, CA).

20

#### Example 1

##### *Purification with DEAE-5PW*

A 25-mer oligonucleotide having the sequence CTCTCGCACCCATCTCTCTCCTTCT (GEM 91) was purified using a 0.5 l column of 30  $\mu\text{m}$  TSK DEAE 5PW (commercially available from TosoHaas). The resin was packed  
25 into a Pharmacia 5.0 cm diameter glass column. Purification was monitored using an ISCO UA-5 detector equipped with a 254 nm filter and a Rainin rabbit pump.

Two sources of GEM 91 were used in this example:

(1) GEM 91 fractions from a Sephadex G-15 column, subsequently desalted and concentrated on Filtron tangential flow filtration (TFF) membranes prior to  
30 chromatography;

(2) two lots of lyophilized GEM 91 powder: one having low ion exchange-HPLC purity, the other having low CGE (capillary gel electrophoresis) purity.

The GEM 91 oligonucleotide was loaded onto the column at 150  $A_{260}$  O.D. (optical density) units/ml packing.

5        25 mM Tris-HCl, pH 7.2 room temperature having from 0.85 to 1.0 M NaCl was used as the buffer with, in some experiments, 1 mM EDTA. Elution was performed with 25 mM Tris-HCl, pH 7.2 (RT) containing 2 M NaCl and, in certain experiments, 1 mM EDTA. The column was washed with 6 column volumes (4 column volumes in run no. 4) of the appropriate equilibration buffer after application of sample. Fractions were  
10 collected, O.D. recoveries determined, and aliquots analyzed for purity by ion exchange HPLC (IEX) and, in some cases, by CGE analysis.

In addition, to runs done on the 0.5 l column, three experiments were done at a 9 ml column-scale in order to evaluate the effect of column configuration on performance.

Results are shown in Table 1. Percent purity was analyzed by ion exchange (IEX)  
15 HPLC (% purity = % of phosphorothioate, including N and N-1 sequences); CGE data is also presented for run #5 (% purity = % of N oligonucleotide, including both phosphorothioate and phosphodiester oligos).

Three experiments were conducted on a 9 ml (2.2 cm diam. x 2.4 cm ht.) column. The results are shown in Table 2.

20        The results demonstrate that crude oligonucleotides can be purified with DEAE SPW with high purity and excellent yield.

Table 1

Run No.	Conditions	Purified pool		
		% recovery O.D. <sup>1</sup>	% purity	% recovery product <sup>2</sup>
1	equil: 0.85M NaCl + EDTA load: 90% IEX purity <sup>3</sup>	54.1	99.6	59.7
5 2	equil: 1.0M NaCl/+ EDTA load: 89% IEX purity	85.8	98.6	94.0
3	equil: 1.0M NaCl load: 89% IEX purity	86.0	99.2	96.2
4	equil: 1.0M NaCl load: 89% IEX purity	58.7	98.0	64.7
5 5	equil: 1.0M NaCl load: 89% IEX purity, 77% CE purity on retests	87.0	97.8 84.7	84.9 <sup>4</sup>
6	equil: 1.0M NaCl load: 87% IEX purity on retest	78.3	98.2	90.1
10 7	equil: 1.0M NaCl load: 87% IEX purity on retest	78.7	98.6	89.3
8	equil: 1.0M NaCl load: 88% IEX purity on retest	83.5	98.4	93.4

<sup>1</sup> percent recovery based on total O.D. (optional density) units initially loaded on column.

<sup>2</sup> percent recovery of product in terms of "purity units" initially loaded on column.

<sup>3</sup> IEX purity units = (total O.D. units) x (percent purity by IEX-HPLC analysis); CE  
15 purity units = (total O.D. units) x (percent purity by CE analysis). CE purity units  
indicate the recovery efficiency of the column.

<sup>4</sup> based on CE data.

Table 2

	[NaCl] in equil. buffer	percent recovery O.D. (total)	Purified Pool		
			% recovery O.D.	% purity (IEX- HPLC)	% recovery of product
9	0.85 M	109.0	87.0	97.0	94.0
10	0.85 M	101.0	80.0	97.0	86.0
11	1.0 M	113.0	67.0	99.0	73.0

5

**Example 2***Purification by Hydrophobic Interaction Chromatography*

A GEM 91 sample was subjected to hydrophobic interaction chromatography. This sample had the composition shown in Table 3.

Table 3

10	percent DMT-on	percent PO	oligonucleotides by CGE analysis			
			n	n-1	n-2	n-3
	62	0.4	55.2	11.4	2.8	3.5

This oligonucleotide was purified on Phenyl-sepharose fast flow (high substitution) using an Amicon 2.2 cm glass column, a Waters 650 solvent delivery system, an HP integrator, a Rainin Dynamax UV-C absorbance detector and a Perkin-Elmer spectrophotometer.

The resin was packed into one of two column configurations: a height:diameter ratio of 2.2:1 or 2.5:1.

The following elution protocols were employed:

(A) the sample load was adjusted to 1.7 M ammonium acetate, pH 10.7; the column was equilibrated and washed with 2.5 M ammonium acetate; sample was eluted using 0.1 M ammonium acetate, pH 8.5, followed by water;

(B) the sample load was adjusted to 1.0 M ammonium acetate, pH 10.7; the column was equilibrated and washed with 1.0 M ammonium acetate, pH 8.5; the sample was eluted with water;



(C) the sample load was adjusted to 1.0 M ammonium acetate, pH 10.7; the column was equilibrated and washed with 1.0 M ammonium acetate, pH 8.5; the sample was eluted using 0.01 M NaOH, pH 11.5, followed by water;

(D) the sample load was adjusted to 0.75 M ammonium acetate pH, 11.0; the column was equilibrated and washed with 0.75 M ammonium acetate, pH 8.5; the sample was eluted using 0.01 N NaOH, pH 11.5, followed by water.

Aliquots of wash and elution fractions were analyzed by ion exchange and reverse phase (RP) HPLC. The results are shown in Table 4.

Table 4

10	Sample Height : Load <sup>1</sup> diameter ratio			Velocity (cm/hr) <sup>2</sup>	Elution protocol	Percent recovery OD <sup>3</sup>		Percent recovery product <sup>4</sup>		Elution purity <sup>5</sup>
						Elution	Total	Elution	Total	
15	1	2.2:1	252	316	A	53.3	99.2	64.0	86.4	97.8
	2	2.2:1	252	316	B	66.1	101.7	96.0	102.0	80.5
	3	2.2:1	252	316/158	C	50.9	98.8	80.8	93.8	88.0
	4	2.5:1	200	316/158	C	55.8	100.8	80.5	85.7	89.8
	5	2.5:1	200	316/158	C	62.7	99.2	84.8	91.1	83.8
	6	2.5:1	200	316/158	C	63.9	100.0	79.7	88.2	77.3
	7	2.5:1	200	316/158	D	59.1	98.3	82.3	86.3	86.3
	8	2.5:1	178	316/158	D	66.1	95.3	83.9	85.9	87.7
	9	2.5:1	178	316/158	D	57.9	89.8	76.6	80.1	91.1

20 <sup>1</sup> Total OD units loaded/ml packing.

<sup>2</sup> Samples 3-9 were loaded at 316 cm/hr and eluted at 158 cm/hour.

<sup>3</sup> % Recovery OD = % recovery of oligonucleotides loaded on column

Elution = % recovery in elution pool

Total = % recovery in *all* fractions

25 <sup>4</sup> % Recovery product = % recovery of "DMT-on" product loaded on column

Elution = % recovery "DMT-on" product in elution pool

Total = % recovery "DMT-on" product in *all* fractions

<sup>5</sup> Indicated in terms of percentage of DMT containing material (% DMT-on); calculated as (% DMT-on by ion exchange HPLC + % DMT-on by reverse phase HPLC)/2.

An ammoniacal solution of GEM 91 having an average of 62% DMT-on was purified on phenyl sepharose fast flow (high substitution) using a Waters 650 solvent  
5 delivery system, a HP integrator, a Rainin Dynamax UV-C absorbance detector, 1 cm (Biorad) and 2.2 cm (Amicon) diameter glass columns and a Perkin-Elmer spectrophotometer.

The deblock solutions were adjusted to 1.7 M ammonium acetate pH 10.3 before loading onto phenyl sepharose. The phenyl sepharose was previously equilibrated with  
10 ammonium acetate. Elution of the oligonucleotide was accomplished with reverse gradients of ammonium acetate, pH 7.85, followed by water. The following run conditions were used:

(A) the column was equilibrated and washed with 2.5 M ammonium acetate, pH 8.5; oligonucleotide eluted with step gradient of 8% ACN in 0.1 M ammonium  
15 acetate pH 7.85, followed by water;

(B) the column was equilibrated and washed in 1.7 M ammonium acetate, pH 8.5; oligonucleotide was eluted with consecutive linear gradients of 1.5 M-1.0 M and 1.0 M-0 M ammonium acetate, followed by water;

(C) the column was equilibrated and washed with 7 M ammonium acetate, pH 8.5; oligonucleotide was eluted with step gradient of 0.5 M ammonium acetate, followed  
20 by linear gradient of 0.5-0 M ammonium acetate, followed by water

(D) the column was equilibrated and washed with 2.6 M ammonium acetate, pH 8.5; oligonucleotide was eluted with step gradient of 0.5 M ammonium acetate, followed by a linear gradient of 0.5-0 M ammonium acetate, followed by water.

25 Aliquots of wash and elution fractions were analyzed by ion exchange and reverse phase HPLC. The results are shown in Table 5.

Table 5

	height: diameter ratio	Load <sup>1</sup>	linear velocity (cm/hr)	elution protocol	<u>recovery of OD</u> (%)		<u>recovery of product</u> (%)		elution purity <sup>2</sup>
					Elution	Total	Elution	Total	
5	5.4:1	192	462	A	46.9	106.4	75.4	78.1	99.7
	2.2:1	385	315	B	42.4	97.7	66.7	83.6	97.8
	2.2:1	385	315	C	18.9	101.5	30.2	93.1	99.0
	2.2:1	252	315	D	32.3	96.6	52.5	85.3	97.5

<sup>1</sup> OD units/ml packing.

10 <sup>2</sup> percent DMT-on: (% DMT-on by ion exchange by ion exchange HPLC + % DMT-on by reverse phase HPLC)/2.

These results demonstrate DMT-on oligonucleotides can be purified from ammoniacal solutions and DMT-off oligonucleotides.

### Example 3

#### 15 *HIC as a Substitute for Rotoevaporation and RPLC*

Two GEM 91 samples were subjected to hydrophobic interaction chromatography. These samples had the composition shown in Table 6. Sample 3-1 had been stored at 4°C as an ammoniacal solution; sample 3-2 had been recently prepared.

Table 6

20	Sample	%DMT-on	%PO	CGE			
				n	n-1	n-2	n-3
	1	63	0.4	56.5	10.3	2.8	2.9
	2	69	0.4	57.6	6.5	2.0	3.7

25 These oligonucleotides were purified on Phenyl-sepharose fast flow (high substitution) using Amicon 2.2 cm glass columns, a Waters 650 solvent delivery system, an HP integrator, a Rainin Dynamax UV-C absorbance detector, and a Perkin-Elmer spectrophotometer.

Ammoniacal solutions of GEM 91 were adjusted to 0.75 M ammonium acetate and loaded at 75 cm/hr onto the column which was previously equilibrated with 0.75 M ammonium acetate, pH 10.2. After loading, the column was washed at 317 cm/hr with (0.75 M) ammonium acetate and DMT-on product was eluted by washing the column at 159 cm/hr with water.

Aliquots of wash and elution fractions were analyzed using ion exchange and reverse phase HPLC. The results are shown in Table 7. The purity and yield of the eluted product is equivalent to that achieved using reverse phase liquid chromatography. This demonstrates that by proper adjustment of load and elution conditions, high purity DMT-on product can be obtained with excellent yield on a relatively short (low height:diameter ratio) chromatography columns with HIC.

Table 7

oligo sample	Load <sup>1</sup>	% recovery of OD		% recovery of product		purity of elution % DMT-on <sup>2</sup>
		Elution	Total	Elution	Total	
1	200	55.8	102.1	85.0	85.3	97.5
1	300	48.5	104.1	73.5	88.8	98.5
1	200	56.6	96.1	83.5	88.9	96.0
2	200	64.0	100.4	89.0	94.3	96.0

<sup>1</sup> total OD units loaded/ml packing.

<sup>2</sup> Purity calculated as: (% DMT-on by IEX-HPLC + % DMT-on by RP-HPLC)/2.

#### Example 4

##### *Oligonucleotide Purification by HIC*

GEM 91 was purified on TSK-gel phenyl-5PW (TosoHaas) and an agarose based phenyl sepharose fast flow (high substitution) (Pharmacia) using a Biorad glass column (1.5 cm diam.), an ISCO UA-5 detector equipped with a 254 nm filter, a Rainin rabbit pump, and a Perkin-Elmer spectrophotometer.

The GEM 91 (sodium salt form) was recovered from desalt column (gel filtration) side fractions from production lots via tangential flow filtration (TFF) on 2,000 MWCO modified polyether sulfone filters (Filtron, Inc.). The oligodeoxynucleotide solutions

were adjusted to 3M NaCl in 25 mM Tris-HCl, pH 7.4-7.5, prior to application to the columns.

**Experimental Conditions:**

A. Phenyl 5PW; oligonucleotide loaded in 3M NaCl/25 mM Tris-HCl pH 7.5,  
5 oligo eluted using linear gradient of 3M - 0M NaCl.

B. Phenyl sepharose; same oligonucleotide loading and elution conditions as in A.

C. Phenyl sepharose; oligonucleotide loaded in 3M NaCl/25 mM Tris-HCl, pH 7.4; oligonucleotide eluted using step gradient of 2M and 1M NaCl followed by linear  
10 gradient of 1M - 0M NaCl.

D. Phenyl sepharose; oligonucleotide loaded in 3M NaCl/25 mM Tris-HCl pH 7.4; oligonucleotide eluted using step gradient of 1M NaCl followed by linear gradient of 1M - 0.7M NaCl, followed by step gradients of 0.7M and 0M NaCl;

The results for each column are shown in Table 8. These results demonstrate that  
15 the sodium salt form of an oligonucleotide can be purified using either phenyl sepharose fast flow resin or phenyl 5PW resin.

**Table 8**

	OD units		Product Recovery, IEX (%) <sup>1</sup>			Product Recovery, CGE (%) <sup>2</sup>		
	elution recovery (%)	recovery: combined fractions (%)	elution recovery (%)	elution purity (%)	recovery: combined fractions (%)	elution recovery (%)	elution purity (%)	recovery: combined fractions (%)
A	53.6	94.5	59.2	95.9	91.7	nd	nd	nd
B	74.8	100.8	77.0	95.6	95.8	77.3	91.8	96.3
20 C	62.5	93.0	65.2	94.9	90.1	63.9	91.7	96.7
D	66.0	113.9	68.3	96.1	112.2	67.1	92.0	110.1

<sup>1</sup> [(number of CGE units in fraction)/(number of CGE units in load)] x 100.

<sup>2</sup> [(number of IEX units in fraction)/(number of IEX units in load)] x 100.

**Example 5**

An ammoniacal solution of GEM 91 having an average of 62% DMT-on was purified on phenyl sepharose fast flow (high substitution) using a Waters 650 solvent delivery system, a HP integrator, 1 cm (Biorad) and 2.2 cm (Amicon) diameter glass  
5 columns and a Perkin-Elmer spectrophotometer.

Phenyl-sepharose was packed into one of three column configurations at height:diameter ratios of: 5.4:1, 2.2:1, and 1:1.

Phenyl sepharose columns were equilibrated in ammonium acetate pH 8.5. The run conditions were as follows:

- 10 (A) no adjustment to the GEM 91 ammoniacal solution; the column was equilibrated with 2.5 M ammonium acetate, pH 8.5; oligonucleotide was eluted with step gradient of 8% acetonitrile (ACN) in water pH 7.85, followed by water; or  
(B) the GEM 91 ammoniacal solution was adjusted to 1.7 M ammonium acetate, pH  
15 pH 7.85; oligonucleotide was eluted with step gradient of 8% ACN in water, followed by water.

Aliquots of wash and elution fractions were analyzed by ion exchange and reverse phase HPLC. The results are shown in Table 9. These results demonstrate that phenyl sepharose fast flow resin can be used to prepare ammoniacal solutions of crude DMT-on  
20 oligonucleotides for subsequent RPLC.

Table 9

	height: diameter ratio	Load <sup>1</sup>	linear velocity (cm/hr)	elution protocol	<u>recovery of OD</u> (%)		<u>recovery of product (%)</u>		elution purity <sup>2</sup>
					Elution	Total	Elution	Total	
5	5.4:1	192	462	A	92.1	103.5	94.4	94.4	68-
	5.4:1	450	462	A	80.8	104.4	97.8	97.8	75
	5.4:1	642	462	A	54.8	100.1	88.5	91.9	80
	5.4:1	1285	462	A	24.5	105.5	nd <sup>3</sup>	nd	nd
	1:1	400	396	A	62.7	101.1	81.4	91.0	81
10	1:1	305	396	A	53.5	98.3	86.3	89.2	72
	1:1	305	157	A	72.4	100.0	87.6	92.4	75
	1:1	250	157	A	80.4	102.0	90.8	91.0	70
	2.2:1	355	396	A	42.1	103.5	55.1	97.2	81
	2.2:1	400	317	B	85.9	105.3	93.5	93.5	68

15 <sup>1</sup> Load: OD units/ml packing

<sup>2</sup> Elution purity: as determined by IEX-HPLC

### Example 6

GEM 91 is synthesized using a CPG support and an automated synthesizer. The oligonucleotide is cleaved from the support using concentrated ammonium hydroxide. The oligonucleotide/ammonium hydroxide mixture is then chromatographed using hydrophobic interaction chromatography using phenyl-sepharose or phenyl-5PW resins essentially according to the procedures set forth above in Examples 1-6. The resulting solutions containing GEM 91 are pooled and optionally chromatographed using preparative reverse-phase liquid chromatography. The combined GEM 91 fractions are acidified to remove the dimethoxytrityl (DMT) protecting group. The detritylated GEM 91 is suspended in water and chromatographed over a DEAE-5PW ion-exchange column essentially as described above in Example 1 to purify it and convert it to the sodium salt form of GEM 91. The oligonucleotide is then "desalted" via tangential flow filtration (TFF) to remove salt and any remaining small molecule impurities, and then depyrogenated on a membrane

referred to as purified BDS. The overall recovery of product after these steps is about 70% with a purity of about 98%. Thus, using this technique, HIC and DEAE 5PW chromatographies can be effectively combined to obtain good recovery of high purity oligonucleotides from ammoniacal solutions.

- 5        From the foregoing, it will be appreciated that although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit or scope of the invention.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Puma Ph.D., Patricia

5 (ii) TITLE OF INVENTION: Purification of Oligodeoxynucleotide  
Phosphorothioates Using DEAE 5PW Anion Exchange Chromatography  
and Hydrophobic Interaction Chromatography

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Chicago  
(D) STATE: IL  
(E) COUNTRY: USA  
15 (F) ZIP: 60606

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
20 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

25 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Greenfield Ph.D., Michael S.  
(B) REGISTRATION NUMBER: 97,142  
(C) REFERENCE/DOCKET NUMBER: 94,444

(ix) TELECOMMUNICATION INFORMATION:

30 (A) TELEPHONE: (312) 715-1000  
(B) TELEFAX: (312) 715-1234

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid

23

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

5

(A) NAME/KEY: misc\_feature  
(B) LOCATION: 1..25  
(D) OTHER INFORMATION: /note= "All internucleotide  
linkages are phosphorothioates"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10 CTCTCGCACC CATCTCTCTC CTTCT

25

**WHAT IS CLAIMED IS:**

1. A method of purifying DMT-off oligonucleotide phosphorothioates and phosphorodithioates comprising applying a mixture of the oligonucleotide to a column containing DEAE-5PW anion exchange chromatography resin and eluting with a salt  
5 containing elution buffer.
2. A method according to claim 1 wherein the oligonucleotide is from 10 to about 35 nucleosides in length.
3. A method according to claim 2 wherein the salt is sodium chloride, which is present in a concentration in the range of about 0 to about 2 M and the pH is between  
10 about 7.0 and about 10.5.
4. A method according to Claim 3, wherein the sodium chloride concentration is from about 0.85 M to about 2 M.
5. A method according to Claim 3, wherein the oligonucleotide phosphorothioates and phosphorodithioates have a length of from about 20-35 oligonucleotides.
- 15 6. A method according to Claim 5, wherein the pH of the elution buffer is about 7.2.
7. A method according to Claim 3, further comprising desalting the DEAE-5PW eluant by tangential flow filtration.
8. A method according to Claim 1 where the oligonucleotide is applied to the  
20 column at a loading concentration of about 150 O.D. units/ml.
9. A method of purifying DMT-off oligonucleotide phosphorothioates and phosphorodithioates comprising applying a mixture of the oligonucleotide to a column containing a hydrophobic interaction chromatography resin and eluting with a salt

10. A method according to claim 9 wherein the hydrophobic interaction chromatography resin is phenyl-sepharose fast flow chromatography resin or phenyl-5PW chromatography resin.

11. A method according to claim 10 wherein the oligonucleotide is from 10 to 5 about 35 nucleosides in length.

12. A method according to claim 11 wherein the salt is sodium chloride, which is present in a concentration in the range of about 1.0 to about 3 M and the pH is between about 7.2 and 8.5.

13. A method according to Claim 12, wherein the sodium chloride concentration 10 is about 3 M.

14. A method according to Claim 12, wherein the oligonucleotide phosphorothioates and phosphorodithioates have a length of from about 20-35 oligonucleotides.

15. A method according to Claim 12, wherein the pH of the elution buffer is about 7.5.

15 16. A method according to Claim 9, further comprising desalting the eluant by tangential flow filtration.

17. A method of purifying DMT-on oligonucleotide phosphorothioates and phosphorodithioates comprising hydrophobic interaction chromatography.

18. A method according to claim 17 wherein the hydrophobic interaction 20 chromatography comprises column chromatography using a phenyl sepharose or TSK phenyl-5PW resin.

19. A method according to claim 17 wherein the oligonucleotides have length of

20. A method according to Claim 18, wherein the salt is ammonium acetate that is present in concentrations of about 0.75 M.

21. A method purifying DMT-on oligonucleotide phosphorothioates and phosphorodithioates comprising removing excess ammonium hydroxide from an  
5 oligonucleotide solution by the method of claim 19.

22. A method of purifying DMT-on oligonucleotide phosphorothioates and phosphorodithioates comprising separating DMT-on oligonucleotides from DMT-off oligonucleotides by the method of claim 19.

23. A method of purifying DMT-on oligonucleotide phosphorothioates and  
10 phosphorodithioates comprising concomitantly removing excess ammonium hydroxide from an oligonucleotide solution and separating DMT-on oligonucleotides from DMT-off oligonucleotides by the method of claim 19.

## INTERNATIONAL SEARCH REPORT

International Application No.

PLI/US 95/08175

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07H1/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 00214 (SEPRACOR INC.) 6 January 1994 see the whole document, but especially, p.56, ex.16 and p.5, 1.4 - p.9, 1.2 ---	1,9,17
A	ANTI-CANCER DRUG DESIGN, vol.6, no.6, 1991 pages 539 - 568 G.ZON ET AL. 'Phosphorothioate Oligonucleotides : Chemistry, Purification, Analysis, Scale-up and Future Directions' see the whole document --- -/--	1,9,17

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \* "A" document defining the general state of the art which is not considered to be of particular relevance
- \* "E" earlier document but published on or after the international filing date
- \* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \* "O" document referring to an oral disclosure, use, exhibition or other means
- \* "P" document published prior to the international filing date but later than the priority date claimed

- \* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \* "&" document member of the same patent family

Date of the actual completion of the international search

26 October 1995

Date of mailing of the international search report

15.11.95

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/08175

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	METHODS IN MOLECULAR BIOLOGY, vol.20, 1993 pages 191 - 206 W.T.WIESLER ET AL. 'Synthesis and Purification of Phosphorothioate DNA' see the whole document ---	1,9,17
A	JOURNAL OF CHROMATOGRAPHY, vol.509, no.2, 22 June 1990 pages 396 - 399 S.AGRAWAL ET AL. 'Analytical Study of Phosphorothioate Analogues of Oligodeoxynucleotides using High-Performance Liquid Chromatography' cited in the application see the whole document ---	1
A	ANALYTICAL BIOCHEMISTRY, vol.200, no.2, 1 February 1992 pages 342 - 346 V.METEV ET AL. 'Ion Exchange High Performance Liquid Chromatography Analysis of Oligodeoxyribonucleotide Phosphorothioates' cited in the application see the whole document ---	1
A	JOURNAL OF CHROMATOGRAPHY, vol.326, 19 June 1985 pages 263 - 280 W.J.STEC ET AL. 'Reversed-Phase HPLC Separation of Diastereomeric Phosphorothioate Analogues of Oligodeoxyribonucleotides and other Backbone-Modified Congeners of DNA' cited in the application see page 277, line 14 - page 279, line 9 -----	1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/08175

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9400214	06-01-94	US-A- 5268097	07-12-93
		AU-B- 4538493	24-01-94
		CA-A- 2138520	06-01-94
		EP-A- 0646038	05-04-95
		US-A- 5393430	28-02-95
		US-A- 5445732	29-08-95
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